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## A New Principle of Thyroxine (T<sub>4</sub>) and Triiodothyronine (T<sub>3</sub>) Radioimmunoassay in Unextracted Serum Using Antisera with Binding Optima at Extreme pH Ranges<sup>1)</sup>

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**Summary:** Thyroxine (T<sub>4</sub>) or triiodothyronine (T<sub>3</sub>) was covalently linked to bovine thyroglobulin (bTg) in a molar ratio of 25:1 (T<sub>4</sub>:bTg) or 30:1 (T<sub>3</sub>:bTg) using a water-soluble carbodiimide. The conjugates of T<sub>4</sub>-bTg and T<sub>3</sub>-bTg were used for immunisation of rabbits. After 6 booster-injections all rabbits immunized with T<sub>4</sub>-bTg had antibody titers from 1:4000 or 1:8000, whereas the rabbits vaccinated with T<sub>3</sub>-bTg had titers from 1:10000 to 1:40000. Two of the T<sub>4</sub>-antisera revealed maximal T<sub>4</sub>-binding between pH 3.8 and 4.2 with affinity constants of  $7.8 \times 10^9$  l/mol and  $6.0 \times 10^9$  l/mol. With these two antisera a simple, rapid, protein-independent T<sub>4</sub> radioimmunoassay at pH 4.0 in unextracted serum was established without using thyroxine binding globulin blocking agents. Precision of the T<sub>4</sub> radioimmunoassay is documented by an interassay coefficient of variation (CV) calculated from 10 different assays of 5.7% at a mean T<sub>4</sub> concentration of 43 µg/l T<sub>4</sub>, 3.5% at 76 µg/l T<sub>4</sub> and 6.4% at 165 µg/l T<sub>4</sub>. The intra-assay CV was calculated to be 3.7%, 2.8% and 4.7% at these concentrations. Accuracy of the T<sub>4</sub>-RIA is documented by identical standard curves when T<sub>4</sub> standards were diluted in 1 g/l human serum albumin in phosphate buffer, in 40 g/l human serum albumin in phosphate buffer, or in T<sub>4</sub>-free serum, and by recovery studies and dilution curves of hyperthyroid patients.

Three T<sub>3</sub> antisera with an optimal binding of T<sub>3</sub> at pH 4 and three T<sub>3</sub> antisera with a pH optimum in the pH range from 8.5 to 10.0 were obtained. A thyroxine binding globulin independent radioimmunoassay for T<sub>3</sub> could be established at pH 9.2 after thyroxine binding globulin in unextracted serum had been denaturated with 0.1 mol/l NaOH. The precision of this T<sub>3</sub> radioimmunoassay is documented by an inter-assay CV (n = 20) of 8.6% at a T<sub>3</sub> concentration of 0.91 µg/l T<sub>3</sub>, 8.5% at a T<sub>3</sub> concentration of 1.720 µg/l T<sub>3</sub> and 5.8% at 4.150 µg/l T<sub>3</sub>. The intra-assay CV calculated from 10 determinations was 5.8%, 3.1% and 3.4% at these T<sub>3</sub> concentrations. Accuracy of the T<sub>3</sub>-RIA is documented by recovery studies, serum dilution curves and identical T<sub>3</sub> calibration curves, when T<sub>3</sub> standards were diluted either in 40 g/l human serum albumin in phosphate buffer or in T<sub>3</sub>-free human serum.

*Ein neues Prinzip für den Radioimmunassay von Thyroxin (T<sub>4</sub>) und Triiodthyronin (T<sub>3</sub>)  
im nicht extrahierten Serum: Verwendung von Antiseren mit Bindungsoptima bei extremen pH-Bereichen*

**Zusammenfassung:** Mit wasserlöslichem Carbodiimid wurde sowohl T<sub>3</sub> als auch T<sub>4</sub> kovalent an Rinder-Thyroglobulin (bTg) in einem Verhältnis von 25:1 (T<sub>4</sub>:bTg) bzw. 30:1 (T<sub>3</sub>:bTg) gekoppelt. Mit diesen Konjugaten wurden je 6 Kaninchen immunisiert. Nach 6 Boosterungen im Abstand von 14 Tagen hatten die Kaninchen, die mit T<sub>4</sub>-bTg immunisiert wurden, ausreichende Antikörper-Titer von 1:4000 bzw. 1:8000, die mit T<sub>3</sub>-bTg immunisierten Kaninchen Antikörper-Titer von 1:10000 bzw. 1:40000. Zwei der T<sub>4</sub>-Antiseren zeigten eine maximale T<sub>4</sub>-Bindung im pH-Bereich von pH 3,8 bis pH 4,2 mit Assoziationskonstanten von  $7,8 \times 10^9$  l/mol bzw.  $6,0 \times 10^9$  l/mol. Mit diesen beiden T<sub>4</sub>-Antikörpern konnte ein einfacher, schneller, Protein-unabhängiger T<sub>4</sub>-RIA bei pH 4,0 im unextrahierten Serum ohne Einsatz von Thyroxin bindendes Globulin blockierenden Substanzen aufgebaut werden. Die Präzision dieses T<sub>4</sub>-Radioimmunoassays konnte mit einem Interassay-Variationskoeffizienten, berechnet aus 10 verschiedenen Bestimmungen von 5,7% bei einer T<sub>4</sub>-Konzentration von 43 µg/l T<sub>4</sub>, 3,5% bei 76 µg/l T<sub>4</sub> und 6,4% bei 175 µg/l T<sub>4</sub> aufgezeigt werden. Der Intraassay-Variationskoeffizient, berechnet aus Zehnfach-Bestimmungen, betrug 3,7%, 2,8% und 4,7% bei diesen T<sub>4</sub>-Konzentrationen. Identische T<sub>4</sub>-Standardkurven wurden erhalten, wenn die T<sub>4</sub>-Standards entweder in 1 g/l Humanserumalbumin in Phosphatpuffer, 40 g/l Humanserumalbumin in Phosphatpuffer oder T<sub>4</sub>-freiem Serum gelöst waren. Damit wurde die Richtigkeit des T<sub>4</sub>-RIA ebenso bewiesen, wie mit Wiederfindekurven und Serumverdünnungskurven, die jeweils auf der Standardkurve lagen.

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Wir erhielten drei  $T_3$ -Antisera mit einem Bindungsoptimum bei pH 4,0 und drei  $T_3$ -Antisera mit einem Bindungsoptimum im Bereich von pH 8,5 bis pH 10,0. Wir konnten einen  $T_3$ -RIA im unextrahierten Serum nach vorheriger Denaturierung von Thyroxin bindendem Globulin mit 0,1 mol/l NaOH und anschließender Inkubation bei pH 9,2 aufbauen. Die Präzision des  $T_3$ -RIA konnte mit einem Interassay-Variationskoeffizienten ( $n = 20$ ) von 8,6% bei einer  $T_3$ -Konzentration von 0,91  $\mu\text{g/l}$   $T_3$ , 8,5% bei einer  $T_3$ -Konzentration von 1,72  $\mu\text{g/l}$   $T_3$  und 5,8% bei 4,15  $\mu\text{g/l}$   $T_3$  gezeigt werden. Der Intraassay-Variationskoeffizient, berechnet aus Zehnfach-Bestimmungen, betrug 5,8%, 3,1% und 3,4% bei den obengenannten  $T_3$ -Konzentrationen. Die Richtigkeit des  $T_3$ -RIA wurde mit Wiederfindungskurven, Serumverdünnungskurven und identischen  $T_3$ -Standardkurven, wobei  $T_3$ -Standards entweder in 40 g/l Humanserumalbumin in Phosphatpuffer oder  $T_3$ -freiem Serum gelöst waren, bewiesen.

## Introduction

The difficulties in radioimmunological determination of thyroxine ( $T_4$ ) and triiodothyronine ( $T_3$ ) in serum are mainly due to the binding of thyroid hormones to specific transport proteins in serum, mainly thyroxine binding globulin (TBG) (1), because the affinity constants of TBG to the thyroid hormones are often in the same order of magnitude as those of the antisera used (2). In recent years many modifications to radioimmunological determinations of thyroid hormones in serum have been published (3, 4, 5, 6). These have all been based upon two principles, using either the somewhat tedious procedure of extraction of  $T_4$  and  $T_3$  from human serum (7) or the more simple method, working with so-called TBG blocking agents mainly 8-anilino-1-naphthalene sulphonic acid (8, 9, 10, 11). These TBG blocking agents, however, also interact with the antisera used in the  $T_3$  and  $T_4$  assay (12), thus rendering this procedure inaccurate, because of varying TBG concentrations in serum. By modification of the immunisation technique we obtained antisera with binding optima for the thyroid hormones at acid or alkaline pH ranges. With these antisera a new type of  $T_4$  and  $T_3$  radioimmunoassay in unextracted serum could be established without the use of TBG blocking agents. Both radioimmunoassays are independent of endogenous TBG concentrations.

## Materials and Methods

### Reagents

Highly purified  $L$ -thyroxine ( $T_4$ ), 3,5,3'-triiodothyronine ( $T_3$ ), 5,3',5'-triiodothyronine ( $rT_3$ ) and tetraiodothyroacetic acid were a gift from the Henning GmbH Berlin. 3,5-diiodotyrosine, 3-monoiodotyrosine, 3,5-diiodothyronine and 3',5'-diiodothyronine were from the Fluka AG, Switzerland, bovine thyroglobulin and hydroxylamine from Sigma Chemicals Co., St. Louis, USA, donkey anti-rabbit-gamma-globulin precipitating serum (second antibody) from Wellcome Research Laboratories, Beckenham, England. Complete Freund's adjuvant, diphtheria-pertussis-tetanus (DPT)-vaccine and human serum albumin from Behring AG, Frankfurt, Germany. Anion exchange cellulose (DE 32) were from Whatman Ltd., Springfield Mill Maidstone, England. All other reagents were p. a. substances from Merck AG, Darmstadt, Germany.

$^{125}\text{I}$ - $T_4$  (specific activity about 3.7 TBq/g (100 Ci/g) was from Hoechst AG, Frankfurt, Germany, and was diluted either in 0.15 mol/l NaCl or in 0.1 mol/l HCl each containing 200 ml/l propylene glycol.

$^{125}\text{I}$ - $T_3$  (specific activity of about 18.5 TBq/g  $\approx$  500 Ci/g) was also from Hoechst AG, Frankfurt, Germany. It was diluted in 0.05 mol/l phosphate buffer containing 1 g/l human serum albumin.

$T_4$ -free serum was pooled from thyroidectomized patients treated longterm with thyrotropin suppressive doses of  $T_3$ .  $T_3$ -free serum was prepared by additional extraction with charcoal.

### Preparation of $T_4$ and $T_3$ conjugates for immunisation

1  $\mu\text{mol}$  of  $T_4$  was dissolved in 100  $\mu\text{l}$  of 0.1 mol/l NaOH and 50  $\mu\text{l}$  dioxan, then 100  $\mu\text{l}$   $^{125}\text{I}$ - $T_4$  (about 20,000 counts/min) purified by Sephadex G-25 chromatography (13), 15 mg bovine thyroglobulin dissolved in 300  $\mu\text{l}$  0.1 mol/l phosphate buffer pH 5.0 and 100  $\mu\text{l}$  of 0.1 mol/l water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for covalent binding of  $T_4$  to bovine thyroglobulin were added. After an incubation time of 24 hours at room temperature, the reaction was stopped by addition of 100  $\mu\text{l}$  0.1 mol/l hydroxylamine (14). The amount of  $T_4$  linked covalently to bovine thyroglobulin (bTg) was calculated from the incorporation of the tracer amount of radioactivity into bTg after dialysis of the solution in a collodium bag (Sartorius Membranfilter GmbH, Göttingen, FRG). The molar ratio estimated was about 25:1 ( $T_4$ :bTg).  $T_3$ -bTg conjugate was obtained by an analogous procedure, the amount of  $T_3$  incorporated to bovine thyroglobulin being approximately 30 mol  $T_3$  per mol of bovine thyroglobulin.

### Immunisation of rabbits

The conjugates of  $T_4$ -bTg and  $T_3$ -bTg were each dissolved in 3 ml 0.15 mol/l NaCl and emulsified with 2 ml of Freund's complete adjuvant. These emulsions were each divided among 6 rabbits both for  $T_4$ -bTg and  $T_3$ -bTg. Sixty to eighty intradermal injections were made into the backs of the rabbits according to the method of Vaitikaitis (15). Each rabbit received a total of 0.8 ml of the emulsion, containing 2.5 mg bTg conjugate. In addition, diphtheria-pertussis-tetanus (DPT)-vaccine, 0.25 ml per rabbit, was injected at a separate site with the first two boosters. Injections were given at two week intervals. After the sixth booster the antibodies were harvested.

### Precipitation procedures of antibody-bound thyroid hormones for radioimmunoassay

Precipitation of antibody-bound  $T_4$  or  $T_3$  for testing antibody titer at a physiological pH-range was performed with polyethylene glycol ( $M_r$  6,000) (16). Polyethylene glycol (250 g) was dissolved in 1000 ml of 0.05 mol/l phosphate buffer pH 8.6. After incubation of 100  $\mu\text{l}$  antiserum dilution with 100  $\mu\text{l}$  of  $T_4$ -tracer (or  $T_3$ -tracer), 100  $\mu\text{l}$  of 1 g/l bovine gamma-globulin in 0.15 mol/l NaCl and 300  $\mu\text{l}$  of the 250 g/l polyethylene glycol solution were added. After vortexing, the tubes were centrifuged at 2000 g for 15 min, the supernatant aspirated off and the precipitate counted for radioactivity.

Because this precipitation method with polyethylene glycol only acts in the neutral pH range (16) and in addition is not specific for rabbit gamma-globulin, the precipitation of antibodies both at different pH ranges and also for radioimmunoassay was performed with a precipitating anti-rabbit-gamma-globulin donkey serum (second antibody) (17). This second antibody

reacts preferably at neutral pH range with specific rabbit antiserum, but it does not dissociate within 24 hours at either pH 3.0 or 11.0 when preincubated at pH 7.4 with the specific antiserum. Binding characteristics of the  $T_4$  and  $T_3$  antisera, which were tested in preliminary experiments, did not change by preincubation with the precipitating antibody. The solution of the complex at pH 7.4 is stable for at least 14 days at 4 °C in 0.15 mol/l NaCl.

#### Titration of the antisera

Antibody titer was calculated from a radioimmunological system at pH 7.4 in phosphate buffer 50 mmol/l. For  $T_4$ -antiserum titration, 100  $\mu$ l of  $^{125}$ I- $T_4$  containing a total of 0.25 pg  $T_4$  diluted in 0.25 mol/l NaCl containing 200 ml/l propylene glycol was added to 100  $\mu$ l antiserum dilution in 1 g/l human serum albumin in phosphate buffer. After an overnight incubation, separation of antibody-bound and free hormone was performed with polyethylene glycol. For  $T_3$ -antiserum, 100  $\mu$ l  $^{125}$ I- $T_3$  containing a total of 0.05 pg  $T_3$  dissolved in 1 g/l human serum albumin in phosphate buffer, was added to 100  $\mu$ l of  $T_3$ -antiserum dilution in 1 g/l human serum albumin in phosphate buffer. The antibody-bound and free hormone separation was performed with polyethylene glycol after an overnight incubation. Antibody titer was defined as the antiserum dilution added per tube which precipitated 50% of radioactivity added per tube.

#### pH-dependency of antibody binding with thyroid hormones

The antisera obtained were tested over a pH-range from pH 3 to 11 for their maximal tracer binding. The antiserum dilution was used which gave 50% tracer binding at pH 7.5. The following incubation scheme was used:

300  $\mu$ l of a buffer solution were added to 100  $\mu$ l of  $^{125}$ I- $T_4$  and 100  $\mu$ l of antiserum dilution preincubated with second antibody, each diluted in 0.15 mol/l NaCl. For the different pH values, 0.1 mol/l citrate buffer was used for pH 3.0 to 7.0, 0.1 mol/l tris-HCl-buffer for pH 7.5 to 9.0 and 0.1 mol/l glycine-NaOH buffer for pH 9.5 to 11.0. For unspecific binding (N) normal rabbit serum instead of specific antiserum was preincubated with second antibody. After an incubation time of 24 hours, when all of the antisera were in equilibrium, the tubes were centrifuged, the precipitate washed with 1 ml 0.15 mol/l NaCl and then counted for radioactivity.

#### Calculation of affinity constants

Affinity constants ( $K_a$ ) of  $T_4$  and  $T_3$  antisera at the different pH-values were calculated from the calibration standard curves by Scatchard analysis (18). The  $T_4$ -standards in the range from 15 to 480  $\mu$ g/l and the  $T_3$ -standards in the range from 0.25 to 8  $\mu$ g/l were dissolved either in 1 g/l human serum albumin in phosphate buffer, 40 g/l human serum albumin in phosphate buffer or  $T_4$ - and  $T_3$ -free human serum.

## Results

### The pH-dependency of thyroid hormone binding of the $T_4$ and $T_3$ antisera

The six rabbits immunised with  $T_4$ -bTg conjugate developed antibody titers from 1:4000 to 1:8000 after 6 booster injections within 15 weeks. The 6 rabbits vaccinated with  $T_3$ -bTg conjugate showed titers from 1:10,000 to 1:40,000.

In respect to maximal  $T_4$ -tracer binding dependent on the pH of incubation buffer, the antisera could be divided into three groups (fig. 1). Two antisera ( $T_4$ -K<sub>4</sub> and  $T_4$ -K<sub>6</sub>) demonstrated an optimal binding between pH 3.8 and 4.2, the antisera  $T_4$ -K<sub>3</sub> and  $T_4$ -K<sub>8</sub> had an optimum between pH 9 and 10, whereas the last group

( $T_4$ -K<sub>5</sub> and  $T_4$ -K<sub>7</sub>) had no definite binding maximum in the pH range tested.

The  $T_4$ -antiserum  $T_4$ -K<sub>5</sub> could be separated on a DE-32 column (10  $\times$  2 cm) into two gamma-globulin fractions using a linear gradient from 0.01 to 0.2 mol/l tris-phosphate buffer pH 8.2. One of the fractions bound most of the  $T_4$ -tracer at pH 4.0 whereas the other bound most of the  $T_4$ -tracer at pH 8.6 to pH 10.0 (fig. 1c).

In contrast to the  $T_4$ -antisera, none of the  $T_3$ -antisera had such a distinct pH dependency. Three of them showed a slight pH-optimum at pH 4, but up to pH 10 the binding of  $T_3$  only fell slightly (to about 85%, taking binding at pH 4.0 as 100%). The remaining three antisera showed the opposite effect, binding maximally at pH 10.

Like the  $T_4$ -antiserum  $T_4$ -K<sub>5</sub>, one of the  $T_3$ -antisera could be separated by DE-32 anion exchange chromatography into two antibody peaks, one with a prominent binding at pH 4, and the other at pH 9.0.

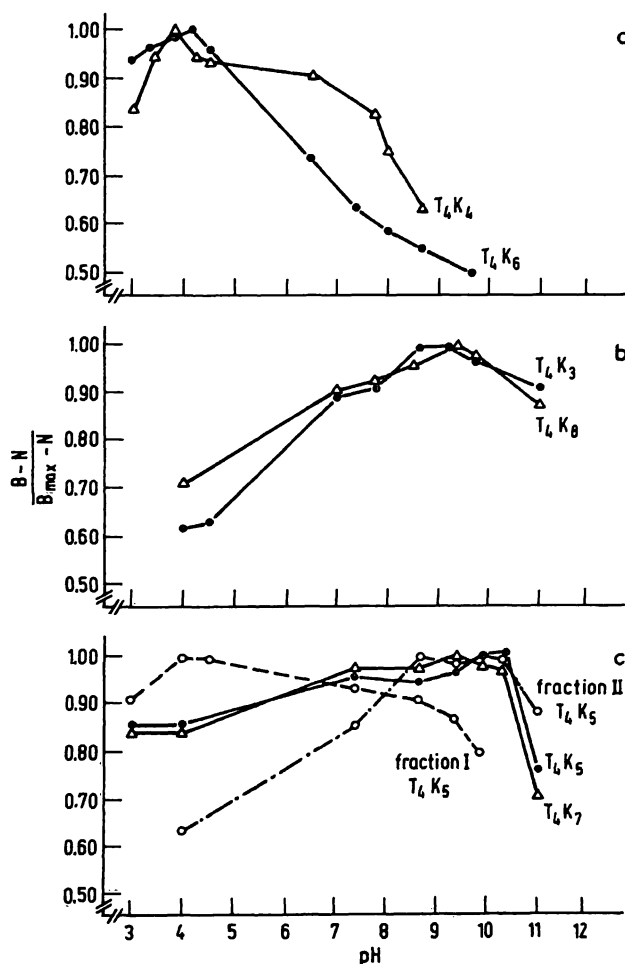


Fig. 1. Dependency of  $T_4$  tracer binding of the  $T_4$  antisera upon the values of the incubation medium. The maximal  $T_4$  binding ( $B_{max} - N$ ) of the antisera in group A was found to be in the range from pH 3.8 to pH 4.2, in group B from pH 9.0 to pH 10.0. Antisera of group C showed no clear cut pH dependency, however when the antiserum  $T_4$ -K<sub>5</sub> was fractionated on a DE-32 column into two antibody fractions, in fraction I the maximal binding of  $T_4$  tracer was found at pH 4.0, whereas fraction II had an optimal binding in the range from pH 8.6 to pH 10.0.

### Affinity constants of T<sub>4</sub> and T<sub>3</sub> antisera

The affinity constants ( $K_a$ ) of T<sub>4</sub> antisera to T<sub>4</sub> and of T<sub>3</sub> antisera to T<sub>3</sub>, respectively, were, as expected, highest in the pH range with maximal tracer binding (tab. 1).

The highest  $K_a$  of the T<sub>3</sub> antisera (T<sub>3</sub>-K<sub>9</sub>, T<sub>3</sub>-K<sub>12</sub>, T<sub>3</sub>-K<sub>13</sub>) with optimal binding between pH 8.0 and pH 9.5 were estimated to be  $5 \times 10^9$  l/mol,  $2 \times 10^{10}$  l/mol and  $6.5 \times 10^9$  l/mol respectively. The three antisera which bound maximally at pH 4.0 had affinity constants at this pH of  $8.5 \times 10^8$  l/mol,  $1 \times 10^{10}$  l/mol and  $1 \times 10^9$  l/mol.

Tab. 1. Dependency of the affinity constants ( $K_a$ ) of four T<sub>4</sub> antisera upon the pH of the incubation buffer.

Antiserum	pH 4.0 $K_a$ (l/mol)	pH 7.4 $K_a$ (l/mol)	pH 10.0 $K_a$ (l/mol)
T <sub>4</sub> -K <sub>4</sub>	$7.8 \times 10^9$	$4 \times 10^7$	$2 \times 10^5$
T <sub>4</sub> -K <sub>6</sub>	$6.0 \times 10^9$	$2 \times 10^7$	$1 \times 10^5$
T <sub>4</sub> -K <sub>3</sub>	$1.0 \times 10^6$	$2 \times 10^7$	$2 \times 10^9$
T <sub>4</sub> -K <sub>8</sub>	$2.0 \times 10^5$	$8 \times 10^6$	$3.5 \times 10^9$

### Specificity of T<sub>4</sub> and T<sub>3</sub> antisera

The two T<sub>4</sub> antisera, T<sub>4</sub>-K<sub>4</sub> and T<sub>4</sub>-K<sub>6</sub>, with binding optima at pH 4.0, and one T<sub>3</sub> antiserum, T<sub>3</sub>-K<sub>12</sub>, with an alkaline pH optimum, later used for a routine radioimmunoassay, were tested for crossreactivity with thyronine analogues, diluted in 1 g/l human serum albumin in phosphate buffer. As summarized in table 2 these antisera revealed high specificity.

Tab. 2. Crossreactivity of two T<sub>4</sub> antisera (T<sub>4</sub>-K<sub>4</sub> and T<sub>4</sub>-K<sub>6</sub>) and T<sub>3</sub> antiserum (T<sub>3</sub>-K<sub>12</sub>) with thyronine derivatives. The crossreactivity of the two T<sub>4</sub> antisera was found to be identical.

Compound	Crossreactivity	
	in T <sub>4</sub> -RIA (%)	in T <sub>3</sub> -RIA (%)
L-Thyroxine	100	0.001
L-Triiodothyronine	0.1	100
Reverse triiodothyronine	3	0.003
Tetraiodothyroacetic acid	12	0.01
Diiodotyrosine	0.3	0.4
Monoidotyrosine	0.001	0.002
3',5'-Diiodothyronine	0.04	0.0004

### T<sub>4</sub>-radioimmunoassay in unextracted serum

Using these T<sub>4</sub>-antisera with optimal binding at pH 4.0, a radioimmunoassay for routine purposes with 10  $\mu$ l unextracted serum per incubation tube at this acid pH was developed without using TBG blocking reagents. The incubation schedule is summarized in table 3. Because of the high concentration of T<sub>4</sub>, the antiserum dilution had to be only 1:800 per tube using 10  $\mu$ l serum (calibration standard) to give a sensitive standard curve with a 50%-intercept at about 60  $\mu$ g/l T<sub>4</sub>.

Tab. 3. Incubation scheme of the T<sub>4</sub>-RIA.

10 $\mu$ l T <sub>4</sub> calibration standard (from 15 $\mu$ g/l T <sub>4</sub> to 480 $\mu$ g/l T <sub>4</sub> ) in 0.05 mol/l phosphate buffer pH 7.4 containing 40 g/l human serum albumin
or 10 $\mu$ l serum
200 $\mu$ l <sup>125</sup> I-T <sub>4</sub> in 0.1 mol/l sodium citrate buffer pH 3.8 containing 200 ml/l propylene glycol
100 $\mu$ l T <sub>4</sub> antiserum (1:800) preincubated with second antibody (1:24), both diluted in 0.15 mol/l NaCl.

After an incubation time of 2 hours, when the reaction was at equilibrium, tubes were centrifuged for 10 min at 2000 g, the precipitate washed with 1 ml of 0.15 mol/l NaCl and after a further centrifugation the precipitate was counted for radioactivity. Calibration curves were calculated by spline function (19).

### Quality control of T<sub>4</sub>-radioimmunoassay

Precision of this assay is documented by the data summarized in table 4. Accuracy of T<sub>4</sub>-radioimmunoassay was shown by independency of different protein contents of the samples: Identical standard curves were found when T<sub>4</sub>-standards were diluted either in 1 g/l human serum albumin in phosphate buffer, 40 g/l human serum albumin in phosphate buffer or T<sub>4</sub>-free human serum with a thyroxine binding globulin concentration of 18 mg/l (fig. 2). Furthermore, when sera of five hyperthyroid patients were diluted in 1 g/l human serum albumin in phosphate buffer, the T<sub>4</sub>-values were on the standard curve (fig. 3). The recovery of a T<sub>4</sub>-standard (80  $\mu$ g/l) added to normal sera (n = 10) was found to be 98%. Total serum T<sub>4</sub>-values determined in this radioimmunoassay (n = 32) of sera with different thyroxine binding globulin concentrations from 2.5 to 68 mg/l were found to be identical (r = 0.985; n = 32) when compared with T<sub>4</sub>-concentrations determined by competitive protein binding analysis. In the latter method T<sub>4</sub> is extracted quantitatively from sera with simultaneous chromatography on alkaline Sephadex G-25 columns (7). The normal range of T<sub>4</sub>-concentration in sera of 144 euthyroid patients was calculated to be 45  $\mu$ g/l to 100  $\mu$ g/l and is identical with the normal range of total T<sub>4</sub> which was determined by competitive protein binding analysis (7).

Tab. 4. Inter- and intraassay coefficient of variation (CV) of the T<sub>4</sub>-RIA at three different T<sub>4</sub> concentrations in serum (n = 10).

T <sub>4</sub> concentration ( $\mu$ g/l)	Interassay CV (%)	Intraassay CV (%)
43	5.7	3.7
76	3.5	2.8
165	6.4	4.7

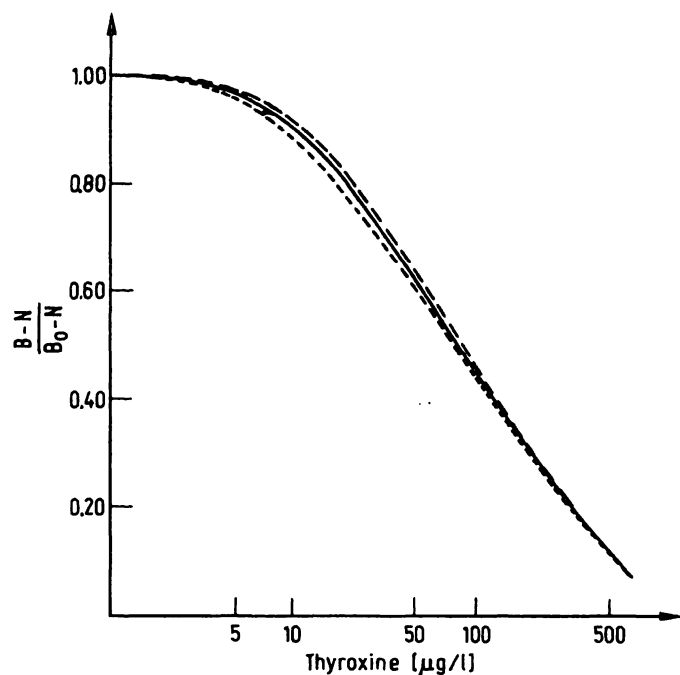


Fig. 2. T<sub>4</sub> calibration curves calculated from Spline function. T<sub>4</sub> standards were either diluted in 1 g/l human serum albumin in phosphate buffer (—), 40 g/l human serum albumin in phosphate buffer (---) or T<sub>4</sub>-free human serum (···) with the thyroxine binding globulin concentration of 18 mg/l.

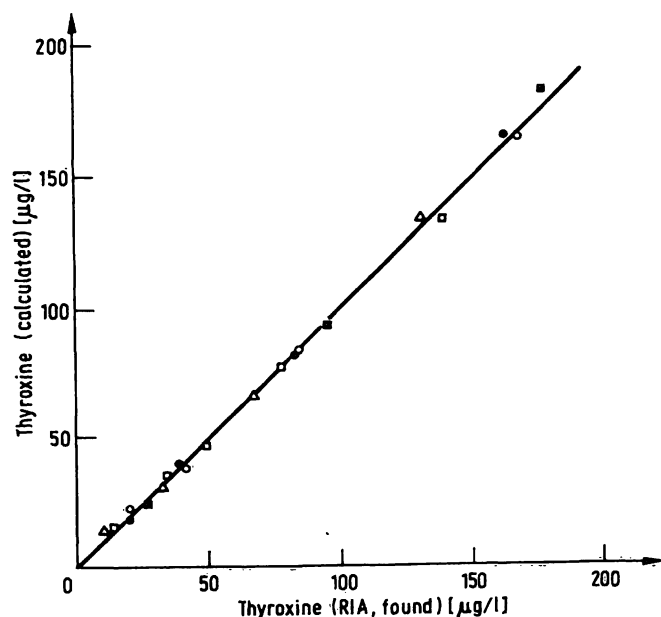


Fig. 3. Serum dilution curves. Sera of five hyperthyroid patients were diluted in a ratio of 1:2, 1:4, 1:8 with 1 g/l human serum albumin in phosphate buffer. T<sub>4</sub> values obtained from the T<sub>4</sub>-RIA were identical to the calculated T<sub>4</sub> values ( $n = 20$ ,  $r = 0.998$ ,  $A = 0.0194$ ,  $B = 995$ ).  $y = -0.0194 + 0.995x$ ,  $r = 0.998$ .

### T<sub>3</sub>-RIA in unextracted serum

With the 3 antisera with a optimum of T<sub>3</sub> binding at pH 4.0 a T<sub>3</sub>-radioimmunoassay was attempted along the lines of the T<sub>4</sub>-radioimmunoassay. These T<sub>3</sub>-antibodies

Tab. 5. Incubation scheme of the T<sub>3</sub>-RIA.

25 µl T <sub>3</sub> calibration standard (from 0.25 µg/l T <sub>3</sub> to 8.00 µg/l T <sub>3</sub> in 40 g/l human serum albumin in phosphate buffer.
or 25 µl serum
200 µl 0.1 mol/l NaOH incubation for at least 5 min
100 µl 1.05 mol/l glycine-NaCl
100 µl <sup>125</sup> I-T <sub>3</sub> in 1 g/l human serum albumin in phosphate buffer
100 µl T <sub>3</sub> antiserum (1:32 000) preincubated with second antibody (1:24) both diluted in 0.15 mol/l NaCl.

were found to bind T<sub>3</sub> related to the different protein concentrations in the samples, as evidenced by serum dilution curves and the recovery of T<sub>3</sub>-standards added to different sera. However, using the T<sub>3</sub>-antisera with optimal binding at alkaline pH, a specific T<sub>3</sub>-radioimmunoassay in unextracted serum could be established. The procedure is shown in table 5. Thyroxine binding globulin in serum is denaturated completely in 0.1 mol/l NaOH within 5 min at room temperature (20); the pH is then adjusted to 9.2 with 1.05 mol/l glycine-NaCl. Preincubated antiserum with the second antibody and the T<sub>3</sub>-tracer was then added. After an incubation time of 4 hours the reaction was in equilibrium and the tubes were centrifuged for 10 min at approximately 2000 g. Standard curves and serum concentrations of T<sub>3</sub> were calculated by Spline function (19).

### Quality control of the T<sub>3</sub>-radioimmunoassay

The precision of the T<sub>3</sub>-radioimmunoassay is documented by the data summarized in table 6. Accuracy of the assay is shown by identical standard curves, when T<sub>3</sub>-standards were either diluted in 40 g/l human serum albumin in phosphate buffer or T<sub>3</sub>-free serum with a thyroxine binding globulin concentration of 18 mg/l (fig. 4). Furthermore, when a T<sub>3</sub>-standard (1.0 µg/l T<sub>3</sub> in 1 g/l human serum albumin in phosphate buffer) was added to human sera with different thyroxine binding globulin concentrations (from 9 mg/l to 23 mg/l) and different endogenous T<sub>3</sub>-levels, the recovery was 100% ( $n = 37$ ;  $r = 0.9985$ ) (fig. 5). When sera of 5 hyperthyroid patients were diluted with 1 g/l human serum albumin in phosphate buffer, the T<sub>3</sub>-concentrations were on the standard curve. The normal range of the T<sub>3</sub>-concentration in sera of 144 euthyroid patients was calculated to be 0.8 µg/l to 1.6 µg/l.

Tab. 6. Inter- and intraassay coefficient of variation (CV) of the T<sub>3</sub>-RIA at three different T<sub>3</sub> concentrations in serum ( $n = 20$ ).

T <sub>3</sub> concentration (µg/l)	Interassay CV (%)	Intraassay CV (%)
0.910	8.6	5.8
1.720	8.5	3.1
4.150	5.8	3.4

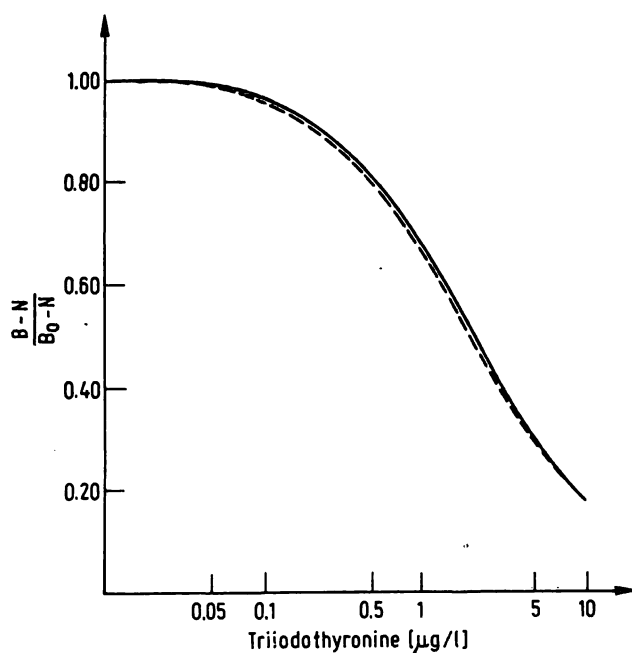


Fig. 4. T<sub>3</sub> calibration curves calculated from Spline function. T<sub>3</sub> standards were either diluted in 40 g/l human serum albumin in phosphate buffer (---), or T<sub>3</sub>-free human serum with a thyroxine binding globulin concentration of 18 mg/l (—).

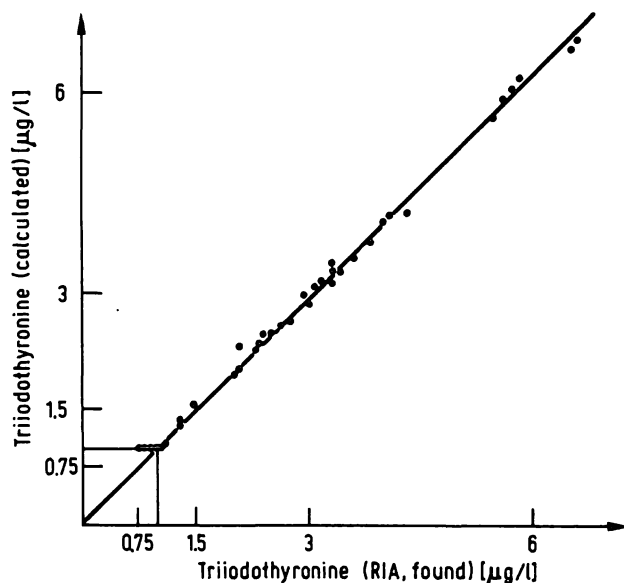


Fig. 5. Recovery study of a T<sub>3</sub> standard added to different human sera.

A T<sub>3</sub> standard (0.1 µg/l T<sub>3</sub> in 1 g/l human serum albumin in phosphate buffer) was added to 37 sera with different thyroxine binding globulin concentrations from 2.5 to 68 mg/l. The recovery was 100% (n = 37; r = 0.9985, A = 9.157, B = 0.958).  
r = 0.9985, A = 9.157, B = 0.958

## Discussion

Thyroglobulin is known to be a very suitable antigen for the production of specific antisera for linked haptens (14). Immunisation with isolated thyroglobulin which physiologically contains covalently bound T<sub>4</sub> and T<sub>3</sub> resulted in low titered T<sub>4</sub> and T<sub>3</sub> antisera (21). This

study has demonstrated that monospecific antisera with sufficient titers can be obtained by immunisation with thyroglobulin enriched with covalently bound exogenous T<sub>4</sub> or T<sub>3</sub> respectively. Some of these antisera demonstrated the ability to bind the antigens with high affinity constants at either acidic or alkaline pH. This phenomenon may be explained by the mode of immunisation, using a high content of haptens coupled to a large glycoprotein molecule, in this case thyroglobulin, and short interval boosters resulting in multiclinal antibodies with different characteristics. The distribution of the antibody populations may vary widely in each of the inoculated animals. The individual selection of antibody clones with pronounced pH dependency was more distinct in the rabbits immunized with T<sub>4</sub>/bTg conjugate compared with those immunized with the T<sub>3</sub>-bTg conjugate.

Until now, T<sub>4</sub> and T<sub>3</sub> radioimmunoassay in unextracted serum could be only performed by using thyroxine binding globulin blocking agents such as 8-anilino-1-naphthalene sulphonic acid and merthiolate (6). These agents showed a displacement of T<sub>4</sub> and T<sub>3</sub> not only from thyroxine binding globulin but also from the antibodies (12), therefore the accuracy of these assays may be influenced by the differences in endogenous thyroxine binding globulin concentrations in the serum samples.

As demonstrated in the last external quality control survey (20) compared to the survey in 1976 (21), the quality of T<sub>4</sub>-RIA kits has been improved. However, using two commercial T<sub>4</sub>-RIA kits (Gamma Coat, Travenol and <sup>125</sup>I-T<sub>4</sub>-RIA, Poemix, Biosigma) and sera containing diminished thyroxine binding globulin (TBG) levels, we found high T<sub>4</sub> concentrations with elevated T<sub>4</sub>/TBG ratios, which disagreed with clinical findings and thyroliberin stimulation tests. Therefore dilutions of sera with elevated, normal and diminished thyroxine binding globulin were made from the T<sub>4</sub>-free serum provided with the kits. Whereas T<sub>4</sub> dilution curves of sera with normal and elevated thyroxine binding globulin levels yielded satisfactory results, the sera of patients with diminished thyroxine binding globulin levels gave inaccurate T<sub>4</sub> dilution curves (tab. 7). This

Tab. 7. Determination of total T<sub>4</sub> in sera with different thyroxine binding globulin (TBG) levels undiluted and in dilutions with T<sub>4</sub>-free serum, using two commercial T<sub>4</sub>-RIA kits (kit A: Gamma Coat, Travenol, kit B: <sup>125</sup>I-T<sub>4</sub>-RIA-Poemix, Biosigma).

	TBG levels (mg/l)	Elevated		Normal		Diminished	
		39	34	20	19	4	6
Kit A	T <sub>4</sub> undiluted	185	168	102	116	61	30
	(µg/l) 1:2	111	93	56	62	18	35
	1:4	63	49	36	38	18	22
Kit B	T <sub>4</sub> undiluted	201	172	95	115	52	21
	(µg/l) 1:2	102	93	49	54	14	30
	1:4	60	48	31	32	12	19

demonstrates dependence of T<sub>4</sub> analytical results on the endogenous thyroxine binding globulin levels in these methods. As demonstrated by Gershengorn et al. (20), thyroxine binding globulin irreversibly changes its tertiary structure at a pH lower than pH 4.5 and above pH 11.5, and the ability to bind thyroid hormone is lost. With the availability of T<sub>4</sub> antibodies with a binding optimum at pH 4.0, a simple, specific and protein-independent radioimmunoassay could be established, because the endogenous T<sub>4</sub> binding proteins showed no influence on T<sub>4</sub> antibody binding in this pH range. This is documented by identical standard curves of T<sub>4</sub> diluted either in 1 g/l human serum albumin in phosphate buffer, 40 g/l human serum albumin in phosphate buffer or T<sub>4</sub>-free human serum with the endogenous thyroxine binding globulin concentrations of 18 mg/l thyroxine binding globulin, by recovery studies and by dilution curves of sera with different thyroxine binding globulin concentrations.

In rabbits, the selection of T<sub>3</sub> antibodies with a binding optimum in an acid pH range was less pronounced compared with the T<sub>4</sub> antisera. A T<sub>3</sub> radioimmunoassay in unextracted sera at pH 4.0 according to the T<sub>4</sub>-RIA without prior separation of the T<sub>3</sub>-antibody populations by anion exchange chromatography, did not fulfill criteria of accuracy. However, using the T<sub>3</sub> antisera with alkaline binding optimum, a thyroxine binding globulin independent T<sub>3</sub> radioimmunoassay could be established without thyroxine binding globulin blocking agents, after a prior denaturation of thyroxine binding globulin with 0.1 mol/l NaOH.

Preincubation of the specific antibody with precipitating antibody (second antibody) did not influence the characteristics of the specific antibody. This procedure is necessary when the radioimmunoassay is performed at extreme pH ranges where the second antibody does not bind the specific antibody; it also represents a simplification of the assay procedure.

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